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Reciprocal Regulation of Protein Kinase C Isoforms Results in Differential Cellular Responsiveness

Raki Sudan,* Neetu Srivastava,* Surya Prakash Pandey,* Subrata Majumdar,† and Bhaskar Saha*

Immunological homeostasis is often maintained by counteractive functions of two different cell types or two different receptors signaling through different intermediates in the same cell. One of these signaling intermediates is protein kinase C (PKC). Ten differentially regulated PKC isoforms are integral to receptor-triggered responses in different cells. So far, eight PKC isoforms are reported to be expressed in macrophages. Whether a single receptor differentially uses PKC isoforms to regulate counteractive effector functions has never been addressed. As CD40 is the only receptor characterized to trigger counteractive functions, we examined the relative role of PKC isoforms in the CD40-induced macrophage functions. We report that in BALB/c mouse macrophages, higher doses of CD40 stimulation induce optimum phosphorylation and translocation of PKCα, βI, βII, and ε whereas lower doses of CD40 stimulation activates PKCθ, ζ, and λ. Infection of macrophages with the protozoan parasite *Leishmania major* impairs PKCα, βI, βII, and ε isoforms but enhances PKCθ, ζ, and λ isoforms, suggesting a reciprocity among these PKC isoforms. Indeed, PKCα, βI, βII, and ε isoforms mediate CD40-induced p38MAPK phosphorylation, IL-12 expression, and *Leishmania* killing; PKCθ and ζ/λ mediate ERK1/2 phosphorylation, IL-10 production, and parasite growth. Treatment of the susceptible BALB/c mice with the lentivirally expressed PKCθ-specific short hairpin RNA significantly reduces the infection and reinstates host-protective IFN-γ-dominated T cell response, defining the differential roles for PKC isoforms in immune homeostasis and novel PKC-targeted immunotherapeutic and parasite-derived immune evasion strategies. The Journal of Immunology, 2012, 188: 2328–2337.

Protein kinase C (PKC), first described as serine/threonine kinases by Nishizuka and colleagues (1), has several isoforms that are classified based on their structure and cofactor requirement into three subfamilies: classical PKCs (PKCα, βI, βII, and γ), novel PKCs (PKCθ, ε, η, and ζ), and atypical PKCs (PKCδ, τ, or λ) (2). PKC isoforms are regulated by their phosphorylation and translocation; once phosphorylated, PKC isoforms are rendered catalytically competent and are activated upon their translocation to cell membrane (2–5). Therefore, activation of PKC isoforms is assessed by their phosphorylation and membrane translocation (2–5). Members of the PKC family are activated by membrane receptor-triggered signals, and the activated PKCs are involved in a wide range of crucial cellular functions, implying their indispensability (6–8). Paradoxically, except PKCα, none of the other PKC isoform-deficient mice is embryonically lethal, raising questions about the nonredundancy for these PKC isoforms in physiological regulation of membrane receptor-triggered cellular effector functions (6, 9). Moreover, relative roles for particular cell-expressed PKC isoforms in receptor-triggered cellular functions have never been addressed. Therefore, using CD40 as a model receptor, we address the differential roles for the macrophage-expressed PKC isoforms in receptor-triggered effector functions.

CD40 is a costimulatory receptor expressed by macrophages, B cells, dendritic cells, and endothelial cells and is the only receptor for which reciprocal signaling has been worked out (10–12). CD40 stimulation by anti-CD40 Ab results in differential and reciprocal regulation of p38MAPK, ERK1/2 phosphorylation, and IL-10 and IL-12 production (11). High doses of CD40 stimulation result in phosphorylation of p38MAPK and p38MAPK-dependent IL-12 production, whereas low doses of stimulation result in ERK1/2 phosphorylation and ERK1/2-dependent IL-10 production (11). In *Leishmania major* infection, CD40-associated p38MAPK phosphorylation and IL-12 production are impaired, whereas ERK1/2 phosphorylation and IL-10 production are enhanced (11). Although the roles for PKC in CD40 signaling in B cells are proposed but remain debatable (13–16), a CD40-induced PKC activity is reported in human monocytes (17). PKC isoforms are implicated in *L. major* and *Leishmania donovani* infection (18–21). However, none of these studies has examined in detail the role of individual PKC isoforms with respect to membrane receptor signaling and how *Leishmania* affects these PKC isoforms. Therefore, the role of PKC isoforms in CD40 signaling in macrophages remains to be addressed.

In this study, we use the transmembrane receptor CD40 and *L. major* infection as models and examine the novel roles for PKC isoforms in CD40 signaling. We report that in BALB/c-derived peritoneal macrophages higher doses of CD40 stimulation induce optimum phosphorylation and translocation of PKCα, βI, βII, and ε, whereas lower doses of anti-CD40 activate PKCθ, ζ, and λ. Infection of macrophages with *L. major* impairs PKCα, βI, βII,
and ε isoforms but enhances PKCδ, ζ, and λ isoforms. PKCα, βI, βII, and ε isoforms induce p38MAPK phosphorylation, IL-12 expression, and Leishmania killing. PKCδ, ζ, and λ promote ERK1/2 phosphorylation, IL-10 production, and parasite growth. Treatment of susceptible BALB/c mice with lentivirally expressed PKCδ- or ε-specific short hairpin RNA (shRNA) significantly reduces infection and reinstates host-protective IFN-γ–dominated T cell response. Collectively, our data define the differential roles for PKC isoforms in CD40 signaling and their implications in a novel PKC-targeted anti-leishmanial immunotherapy.

Materials and Methods

Reagents

Abs specific for p-PKCβII, p-PKCδ, and p-PKCζ/λ (Cell Signaling Technology, Danvers, MA), for p-PKCα (Millipore, Bedford, MA), for p-PKCγ, PKCα, p-PKCβ, PKCβIII, PKCδ, p-PKCε, PKCε, PKCη, PKCζ, PKCa, p-p38MAPK, total p38MAPK, pERK1/2, total ERK1/2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and for Pan-Ras (Pierce, Rockford, IL) were purchased. Anti-cytokine Abs (IL-12, IL-10, IFN-γ, and IL-4) and standard cytokines for ELISA, anti-CD40 Ab (NA/LE; clone 3/23), and isotype control were from BD Biosciences (San Diego, CA). Soluble mouse recombinant CD40L was from Alexis Biochemicals (San Diego, CA); PKCα/β myristoylated pseudosubstrate peptide inhibitor, PKCβ inhibitor, and PKCζ/λ myristoylated pseudosubstrate peptide inhibitor were from Calbiochem (San Diego, CA); cell-permeable myristoylated translocation peptide inhibitors for PKCδ (V1-1; Myr-SFNSYLGLS-OH) and PKCε (Myr-EAVSLKPT-OH) were synthesized from GenPro Biotech (New Delhi, India).

Mice, parasites, and infection

BALB/c mice and CD40-deficient mice (BALB/c background) were originally obtained from (Bar Harbor, ME) and were bred in the National Centre for Cell Science’s experimental animal facility. All animal usage protocols were approved by the Institutional Animal Care and Use Committee. L. major (strain MHOM/Su73/5ASKH) was maintained in vitro in RPMI 1640 medium supplemented with 10% FCS (Life Technologies-BRL, Grand Island, NY) and the virulence was maintained by passage through BALB/c mice. Stationary phase L. major promastigotes (2 × 10^7) were used to s.c. infect BALB/c and CD40-deficient mice in their hind footpad.

In experiments with lentivirus treatment, mice were injected s.c. in the hind footpad (same footpad in which L. major infection was given) with 5 × 10^7 transduction units (in 50 μl HBSS; Life Technologies, Grand Island, NY) of lentivirus expressing PKCδ shRNA, PKCζ shRNA, and control shRNAs on the third day of infection. Some mice were treated with anti-

FIGURE 1. Expression and CD40-induced phosphorylation and membrane translocation of PKC isoforms in uninfected macrophages and L. major promastigote-infected macrophages. (A) BALB/c-derived peritoneal macrophages were either left uninfected or infected with L. major (1 macrophage/10 parasites) for indicated time points and analyzed for expression of PKC isoforms by immunoblotting and RT-PCR. (B) BALB/c-derived peritoneal macrophages were stimulated with isotype control (rat IgG2a, 3 μg/ml), anti-CD40 Ab (3 μg/ml, clone 3/23), or CD40-ligand (CD40L; 80 ng/ml) for 10 min. Macrophages were lysed and assessed for phosphorylation (left panel) and membrane translocation (right panel) of various PKC isoforms by using isoform-specific Abs by immunoblotting. (C) Uninfected macrophages and 12 h L. major-infected macrophages (1 macrophage/10 parasites) were stimulated with indicated doses of anti-CD40 Ab for 10 min and were assessed for phosphorylation of various PKC isoforms by immunoblotting. (D) Uninfected macrophages and 12 h L. major-infected macrophages (1 macrophage/10 parasites) were stimulated with indicated doses of anti-CD40 Ab for 10 min and lysed, membrane fractions were isolated by ultracentrifugation (100,000 × g for 30 min), and macrophages were assessed for membrane translocation of PKC isoforms by immunoblotting. Pan-Ras was used as loading control. The experiments were performed three times, of which one set of representative data is shown. IM, infected macrophages; UIM, uninfected macrophages.
CD40 (i.p., 50 μg/mouse; clone 3/23) for 3 d alternatively beginning day 4 postinfection. Disease severity was assessed by measurement of footpad swelling using a digital micrometer (Mitutuyo Japan) and parasite burden (11, 12).

L. major infection of macrophages

Thioglycolate-elicited BALB/c-derived macrophages were cultured in RPMI 1640 supplemented with 10% FCS and were infected with L. major promastigotes at a 1:10 ratio for 6 h. Extracellular parasites were removed before adding PKC inhibitors and anti-CD40 Ab. The amastigotes were enumerated after 72 h (22, 23).

PKC isoform inhibitors and inhibitor treatment

PKCo/β inhibitor is a myristoylated pseudosubstrate peptide inhibitor (PKC 20–28, myristoyl-FARKGALRQ-OH), which consists of pseudosubstrate peptide sequence derived from PKCo and PKCB, and the N terminus is myristoylated to allow membrane permeability (24). PKCB inhibitor is an anilino-moniodonylemaleimide compound that acts as a potent, cell-permeable inhibitor of PKCB isoforms (25). PKCA/α inhibitor is a myristoylated pseudosubstrate peptide inhibitor (Myr-SIYRRGARRWRKL-OH), which consists of pseudosubstrate peptide sequence derived from PKCA and is a cell-permeable, reversible, substrate competitive inhibitor (26). PKCS (V1-1; Myr-SFNSYLGLS-OH) and PKCC (Myr-EAVSLKPT-OH) inhibitors are cell permeable myristoylated translocation peptides inhibitors of PKCS and PKCC (27, 28). BALB/c peritoneal macrophages were treated with indicated doses of PKC inhibitors for 2 h followed by anti-CD40 Ab treatment for 15 or 10 min for Western blotting or for 8 h for RT-PCR or 48 h for ELISA.

Western blotting

After treatment with the indicated reagents, cells were washed twice with chilled PBS and lysed in cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, protease inhibitor mixture; Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor mixture (Pierce) by incubation on ice for 45 min. Lysates were centrifuged (12000 rpm, 15 mins) and supernatants were collected. Protein was quantified by BCA kit (Pierce) and equal amount of protein was run on SDS–PAGE. Resolved proteins were blotted to PVDF (Millipore) and then blocked with 5% nonfat dried milk in TBST (25 mM Tris [pH 7.6], 137 mM NaCl, and 0.2% Tween 20). Membranes were incubated with primary Ab at 4˚C overnight, washed with TBST, and incubated with HRP-conjugated secondary Ab. Immunoreactive bands were visualized with the luminol reagent (Santa Cruz Biotechnology).

Subcellular fractionation

After treatment with the indicated reagents and time period, cells were washed twice with chilled PBS and were scraped in 1 ml PBS. Cells were collected by centrifugation and were suspended in lysis buffer without Nonidet P-40 (20 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, protease inhibitor mixture, and phosphate inhibitor mixture). The suspension was then sonicated with full power (Vibra Cell, New Town, CT; five times for 10 s each). Nuclei, granules, and unbroken cells were removed by centrifugation at 1,000 g for 10 min at 4˚C. Supernatants were ultracentrifuged at 100,000 g for 30 min at 4˚C (TLA-100 rotor; Beckman Coulter) to separate membrane fractions. Supernatants were the cytosolic fractions, and pellets were the membrane fractions (29), which were washed and resuspended in the lysis buffer containing 1% Nonidet P-40.

Small interfering RNA transfection

P38SD1 macrophage-like cell line was used to carry out small interfering RNA (siRNA) transfection studies. Various PKC isoform-specific siRNA, control siRNA, transfection medium, and transfection reagent were procured from Santa Cruz Biotechnology. siRNA transfection was carried out according to the manufacturer’s protocol. Briefly, 5 × 10⁴ cells were plated 3 h before adding PKC inhibitors and anti-CD40 Ab. The amastigotes were enumerated after 72 h (22, 23).
in a 6-well plate 24 h before transfection. Cells were transfected in serum-free media with 60 picomoles siRNA duplex. After 6 h, complete medium (1 mL RPMI 1640/10% FCS) was added; 12 h later, this medium was replaced with standard culture media. After 36–48 h siRNA transfection, cells were stimulated with anti-CD40 (3 μg/mL) and were harvested for Western blot analysis.

Cytokine ELISA

Culture supernatants from macrophages—uninfected or infected with L. major—after 12 h and treated with various PKC isoform-specific inhibitors followed by anti-CD40 (3 μg/mL) stimulation for 48 h—or lymph node cells stimulated with anti-CD3 (0.5 μg/mL) and anti-CD28 (2 μg/mL) Abs for 60 h from L. major-infected and lentivirally expressed shRNA and anti-CD40-treated BALB/c mice were assayed for IL-10 and IL-12 (p70) (p70) or IFN-γ, respectively. ELISA plates were coated overnight at 4°C with purified anti-IL-10 (2 μg/mL), IL-12 (2 μg/mL), anti-IL-4 (1 μg/mL), or IFN-γ (2 μg/mL). Plates were washed three times (0.05% Tween 20 in PBS) and blocked for 2 h with blocking buffer (1% BSA). Plates were washed three times and then were incubated overnight with standards or culture supernatants. Plates were washed and incubated with respective biotin-conjugated detection Abs for 1 h at 25°C. Plates were then washed and incubated with peroxidase-conjugated streptavidin (Roche Applied Science) for 45 min followed by washing and development with TMB substrate. Reactions were stopped by the addition of 1 N H2SO4, and absorbance was measured at 450 nm (11, 12).

RT-PCR

Total RNA was extracted using TRI reagent (Sigma-Aldrich, St Louis, MO) as per the manufacturer’s guidelines. For cDNA synthesis, 2 μg RNA from each sample was incubated with 0.6 μg random primer in a total volume of 15 μL (Master Mix I) for 5 min at 70°C and then kept on ice. To the above Master Mix I, 10 μL Master Mix II was added containing 1× RT buffer, 8 mM DTT, 400 μM dNTPs, 40 U RNase inhibitor, and 200 U Moloney murine leukemia virus-reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). cDNA synthesis was carried out at 37°C for 1 h followed by heat inactivation of the enzymes at 68°C. Using PCR, cDNA from each sample was amplified with 2.5 μL 10× PCR buffer, 0.75 μL 50 mM MgCl2, 0.5 μL 10 mM dNTP, 0.5 μL each forward and reverse primer, and 0.2 μL (5 U/μL) of Taq DNA polymerase (Invitrogen Life Technologies) for 25 μL reaction. cDNA was amplified under following conditions: 95°C for 2 min, 94°C for 1 min, appropriate annealing temperature for 1 min, and 72°C for 1 min, for a total of 30–35 cycles followed by final extension for 10 min at 72°C. Each sample was amplified for mouse GAPDH to ensure equal cDNA input. Amplified PCR products were analyzed in 1.2% agarose gel.

We used the following primers for RT-PCR: GAPDH forward, 5′-GACCCAAACGGGTCTACATC-3′; reverse, 5′-CCTGCTCTCCACCTTTCTG-3′; inducible NO synthase 2 (iNOS) forward, 5′-CAGAGGACCA-GAGACAAAGC-3′, reverse, 5′-AAAGACAGGGAGCAGCACC-3′; PKCα forward, 5′-AAGACACACTCCAAACAACC-3′; reverse, 5′-TCTCTGGGC-CAATGATGCC-3′; PKCβI forward, 5′-ATGAAACTCAGGATTAAATTAATCA-3′; PKCδ forward, 5′-CACCACCTCCCAGAAAACG-3′, reverse, 5′-CTCGCCATAGGCTCGT-3′; PKCε forward, 5′-CATCTTGATTCGTTGACAGCC-3′, reverse, 5′-CCTCCACGAGTTG-3′; PKCe forward, 5′-CATGGCGTTCTGCAGCTAC-3′, reverse, 5′-CGGTTGGCAATGCAAAAGGC-3′; PKCγ forward, 5′-GCATCAACATCCACAAAGG-3′, reverse, 5′-CGTTCTCTTGCACTGCACCTT-3′; PKCζ forward, 5′-CAATGCTGGATGAGGATTAAAMA-3′, reverse, 5′-GGTGTCATCATCAGAGC-3′; PKCα forward, 5′-CAGCCGATGTTGAAAGGA-AGFAT-3′, reverse, 5′-TCTCTGGCTCTTTGGTAG-3′.

Production of PKCδ shRNA, PKCζ shRNA, and control shRNA lentivirus particles

PKCδ shRNA (GenBank accession no. NM_011103) in pGPZ lentivirus vector, control shRNA in pGIPZ lentivirus vector, PKCζ shRNA (GenBank accession no. NM_001039079) in pLKO.1 lentivirus vector, and control shRNA in pLKO.1 lentivirus vector were purchased from Open Biosystems (Huntsville, AL). Control shRNAs and PKCδ and PKCζ shRNAs were packaged in a Trans-Lentiviral packaging system (replication-incompetent HIV-1–based lentivirus; Open Biosystems) using an HEK293T producer cell line following the manufacturer’s protocol. Briefly, 5.5 × 10⁵ cells were plated in a 100 mm Petri plates 1 d before transfection. Trans-Lentiviral packaging mix (28.5 μg) and PKCδ (9 μg) and PKCζ (9 μg) shRNAs or control shRNA (9 μg) plasmid DNA was mixed together. Transfection was carried out in serum-free media using Arrest-In transfection reagent (Invitrogen) at a 1:5 DNA/transfection reagent ratio. After 6 h, medium was changed with standard culture medium (DMEM supplemented with 10% FCS) and cells were incubated for 72 h. Virus supernatant was harvested after 48 and 72 h, combined, filtered through a 0.45-μm filter (Millipore), and concentrated 100-fold.

In vitro lentiviral transduction

Peritoneal macrophages were transduced with lentiviral (Lv) particles expressing PKCζ shRNA, PKCζ shRNA, and control shRNAs (2 transduction units per cell of each) for 8 h in RPMI 1640 supplemented with 0.5% FCS and 8 μg/mL polybrene (Sigma-Aldrich). Cells were washed to remove residual viral particles. After 48 h transduction, cells were stimulated with anti-CD40 Ab (3 μg/mL) for 15 min for Western blotting or 48 h for ELISA (30).

FIGURE 3. PKC isoforms differentially regulate CD40-induced IL-10 and IL-12 (p70) production in uninfected macrophages and L. major-infected macrophages. (A–E) Twelve hour L. major-infected (1 macrophage/10 parasites) macrophages were treated with 15 μM PKCα/B (A), 7 μM PKCβ (B), 10 μM PKCδ (C), 10 μM PKCε (D), or 15 μM PKCζ/λ (E) inhibitors for 2 h followed by anti-CD40 stimulation (3 μg/mL) for 48 h. Culture supernatants were collected and assessed for IL-10 and IL-12 (p70) production by ELISA. The error bars represent mean ± SEM. Data are from one experiment representative of experiments done at least three times. IM, infected macrophages; UIM, uninfected macrophages.
**Results**

*L. major* differentially alters the expression and CD40-induced activation of PKC isoforms

To check the possible involvement of PKC isoforms in *L. major* infection, we first assessed the expression of all eight macrophage-expressed PKC isoforms (31, 32) in thioglycolate-elicited BALB/c-derived peritoneal macrophages at different time points (12, 24, 36, 48, 60, and 72 h) after *L. major* infection in vitro by Western blotting and RT-PCR. We observed that compared with their expressions in uninfected macrophages, PKCα, βI, and βII expressions were impaired, whereas PKCδ, ε, and λ expressions were enhanced in *L. major*-infected macrophages both at protein as well as at RNA levels (Fig. 1A); the expressions of PKCζ and PKCe remained unaffected (Fig. 1A). This observation corroborates the previously reported impaired PKCζ expression or enhanced PKCζ expression in *L. donovani*-infected macrophages (19). To check the possible involvement of PKC isoforms in CD40 signaling, we studied the CD40-induced phosphorylation and membrane translocation of macrophage-expressed PKC isoforms. Except for PKCζ, all other PKC isoforms were phosphorylated (Fig. 1B, left panel) and translocated (Fig. 1B, right panel) by CD40 stimulation through anti-CD40 Ab or CD40-ligand; the phosphorylation and translocation was not affected by an isotype-matched Ab (Fig. 1B), indicating involvement of PKC isoforms in CD40 signaling in BALB/c peritoneal macrophages. When compared for CD40 dose-dependent phosphorylation and membrane translocation after 12 h *L. major* infection, the time when the expression of any PKC isoforms remained unaltered, PKCα, βI, and ε were phosphorylated (Fig. 1C, Supplemental Fig. 1A) and translocated (Fig. 1D, Supplemental Fig. 1B) at higher doses of anti-CD40, but the extent of phosphorylation and translocation was less or impaired in *L. major*-infected macrophages, whereas PKCδ and PKCζ/λ were phosphorylated and translocated more at a lower dose of anti-CD40 and in the infected macrophages (Fig. 1C, 1D, Supplemental Fig. 1A, 1B). Similar to its expression, phosphorylation and membrane translocation of PKCζ remained unaffected in *L. major*-infected macrophages (Fig. 1C, 1D, Supplemental Fig. 1A, 1B). These data indicate that *L. major* differentially affects the expression and the CD40-induced phosphorylation and translocation of these PKC isoforms.

**PKC isoforms differentially modulate CD40-induced p38MAPK and ERK1/2 phosphorylation**

Because phosphorylation and membrane translocation of PKC isoforms were differentially regulated by the doses of anti-CD40, which also regulates p38MAPK and ERK1/2 phosphorylation differentially (11), we next examined whether the selective inhibition of these PKC isoforms by their inhibitors and siRNAs differentially regulated CD40-induced reciprocal signaling through these two MAPKs. Indeed, the inhibition of PKCα/β, βI, and ε isoforms by their inhibitors reduced the CD40-induced p38MAPK phosphorylation but enhanced ERK1/2 phosphorylation (Fig. 2A, Supplemental Fig. 2A), whereas the inhibition of PKCζ and λ isoforms resulted in a reverse profile of CD40-induced p38MAPK and ERK1/2 phosphorylation (Fig. 2A, Supplemental Fig. 2A). To study the specificity of the PKC inhibitors, we treated BALB/c-derived peritoneal macrophages with these PKC inhibitors for 2 h followed by stimulation with anti-CD40 (3 μg/ml) for 10 min and studied the PKC isoform activation by membrane translocation of various PKC isoforms. We observed that the different PKC inhibitors inhibited the CD40-induced translocation of the PKC isoforms for which they were specific (Fig. 2B). Inhibition of

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**FIGURE 4.** PKC isoforms differentially regulate CD40-mediated amastigote killing and iNOS2 expressions. (A–E) BALB/c-derived peritoneal macrophages were infected with *L. major* promastigotes at a ratio of 1 macrophage/10 parasites for 6 h, extracellular parasites were washed out, and macrophages were treated with indicated doses of PKCα/β (A), PKCζ (B), PKCδ (C), PKCe (D), and PKCζ/λ (E) inhibitors for 2 h followed by anti-CD40 stimulation (3 μg/ml). Macrophages were incubated for 72 h, fixed Giemsa stained, and the number of amastigotes per 100 macrophages was counted microscopically. The experiments were repeated at least three times, and data from one representative experiment are shown. Data shown mean value ± SEM (error bars). *p < 0.01, **p < 0.001. (F–J) Peritoneal macrophages were treated with the indicated doses of PKCα/β (F), PKCζ (G), PKCδ (H), PKCe (I), and PKCζ/λ (J) inhibitors for 2 h followed by anti-CD40 stimulation (3 μg/ml) for 8 h. Macrophages were assessed for iNOS2 expression by RT-PCR. The experiments were repeated at least three times, and data from one representative experiment are shown.
expression of PKCo, β, δ, ε, and ζ isoforms by their siRNAs induced similar effects on CD40-induced phosphorylation of p38MAPK and ERK1/2 as observed in case of PKC inhibitors (Fig. 2C–G, Supplemental Fig. 2B), suggesting that CD40-induced p38MAPK and ERK1/2 phosphorylation are differentially modulated by different PKC isoforms.

**PKC isoforms reciprocally regulate CD40-induced IL-10 and IL-12 production in uninfected and L. major-infected macrophages**

Because CD40-induced IL-12 and IL-10 productions are directly associated with p38MAPK and ERK1/2 phosphorylation, respectively (11, 12), we tested whether PKC isoform-specific inhibitors regulate these two counteractive cytokines differentially in uninfected and L. major-infected macrophages. Inhibition of the PKCo/β, β, and ε isoforms resulted in enhanced CD40-induced IL-10 production but reduced IL-12 production (Fig. 3A, 3B, 3D), whereas the inhibition of PKCδ and ζ/λ isoforms resulted in a reverse profile of CD40-induced IL-10 and IL-12 production (Fig. 3C, 3E). Another novel observation was that the inhibition of PKCδ and ζ/λ isoforms restored the CD40-induced IL-12 production with concurrent inhibition of IL-10 production in L. major-infected macrophages (Fig. 3C, 3E), whereas the inhibition of the PKCo/β, β, and ε isoforms inverted IL-10 and IL-12 productions (Fig. 3A, 3B, 3D). These data suggest that in L. major infection, where the CD40-induced expression of the host-protective proinflammatory cytokine IL-12 is impaired, targeted suppression of PKCδ and ζ/λ isoforms results in host protection.

**PKC isoforms differentially regulate CD40-mediated amastigote killing and iNOS2 expression**

Because host protection is associated with reduced amastigote number in macrophages and enhanced iNOS2 expression (11, 12, 22), we tested the effect of PKCo/β, β, δ, ε, and ζ/λ inhibitors on amastigote number and iNOS2 expression in thioglycollate-elicited BALB/c-derived peritoneal macrophages. We observed that the inhibition of PKCδ and ζ/λ isoforms enhanced the anti-leishmanial activity of CD40, as inhibition of PKCδ and ζ/λ along with anti-CD40 treatment resulted in reduced amastigote count (Fig. 4C, 4E) but increased iNOS2 expression (Fig. 4H, 4I) in L. major-infected macrophages. On the contrary, inhibitors of PKCo/β, β, and ε negated the anti-leishmanial activity of CD40 (Fig. 4A, 4B, 4D) and reduced CD40-induced iNOS2 expression (Fig. 4F, 4G, 4I), indicating that the parasite differentially regulates the reciprocal signaling through PKCo, β, and ε isoforms versus PKCδ and ζ/λ isoforms. Whereas the L. major-inhibited PKCo, β, and ε isoforms promote p38MAPK phosphorylation and IL-12 production, the functional correlates of host protection, the L. major-enhanced PKCδ and ζ/λ isoforms, promote ERK1/2 phosphorylation and IL-10 production, the disease-promoting correlates. These observations suggest a novel parasite-devised finer PKC-targeted immune evasion strategy.

**Lentivirally expressed PKCζ/ζ shRNA differentially modulated CD40-induced ERK1/2, p38MAPK phosphorylation, and IL-10 and IL-12 production in macrophages**

Because we observed in previous results that inhibition of PKCδ and ζ resulted in reduction in CD40-mediated ERK1/2 phos-
phorylation and IL-10 production while enhancing p38MAPK phosphorylation and IL-12 production, we next tested the effect of in vitro silencing of PKCδ or ζ by lentivirally expressed PKCδ and PKCζ shRNAs on CD40-induced p38MAPK, ERK1/2 phosphorylation, and IL-12 and IL-10 production. We observed that inhibition of PKCδ (Fig. 5A, 5B) or PKCζ (Fig. 5C, 5D) resulted in reduction in CD40-mediated ERK1/2 phosphorylation and IL-10 production while reciprocally enhancing p38MAPK phosphorylation and IL-12 production, recapitulating the previous results. We did not observe any change by control shRNA treatment (Fig. 5).

**PKCδ or ζ isomform-targeted therapy enhances anti-leishmanial effects of CD40 in a susceptible host**

Because the parasite differentially modulates the expression, phosphorylation, and translocation of the PKC isoforms for its survival, we examined whether inhibition of PKCδ or PKCζ isoforms can be an anti-leishmanial therapy. Therefore, BALB/c mice infected with the stationary phase *L. major* promastigotes (2 × 10^6 parasites, s.c) were treated once with the PKCδ or PKCζ shRNA Lv particles (5 × 10^5 transduction units) 2 d after the infection, followed by anti-CD40 (50 μg/mouse, i.p) treatment for 3 alternate days. Whereas the progression of the infection was monitored weekly by net footpad swelling (the difference in footpad thickness between the infected and uninfected footpad), the parasite load in the draining lymph node was enumerated 5 wk after the infection. It was observed that the anti-CD40 cotreatment with either PKCδ shRNA or PKCζ shRNA reduced the footpad thickness and the parasite load significantly (p < 0.001) (Fig. 6A, 6B), although treatment with either agent alone (Lv-shRNA or anti-CD40) also significantly reduced the parasite load and footpad thickness (p < 0.01) (Fig. 6A, 6B). The reduction of the parasite load was accompanied with heightened IFN-γ (p < 0.001 for PKCδ shRNA and p < 0.01 for PKCζ shRNA, as compared with control shRNA) and lowered IL-4 responses (p < 0.001 for PKCδ shRNA and PKCζ shRNA, as compared with the control shRNA) (Fig. 6C, 6D). These results indicate that PKC isoform-specific therapy can protect even a susceptible CD40-sufficient host.

To check the effect of PKCδ and PKCζ shRNA Lv treatment on the expression of respective isoforms under in vivo conditions, we performed the Western blots of these isoforms from footpad lesions and lymph node of *L. major*-infected BALB/c mice treated with PKCδ shRNA Lv or PKCζ shRNA Lv or control shRNA Lv. We observed significant reduction in levels of PKCδ and PKCζ in mice treated with shRNA Lv particles for respective isoforms compared with mice treated with control shRNA Lv (Fig. 6A, inset, 6B, inset).

**PKCδ or PKCζ shRNA Lv treatment failed to rescue *L. major* infection in CD40-deficient mice**

Because we observed that PKCδ or PKCζ shRNA Lv treatment alone or along with anti-CD40 treatment significantly reduced *L. major* infection in BALB/c (CD40+/+) mice, we tested whether the

**FIGURE 6.** PKCδ or PKCζ isomform-targeted immunotherapy protects susceptible BALB/c mice from *L. major* infection. (A and B) BALB/c mice infected s.c. on one hind footpad with 2 × 10^6 *L. major* promastigotes were treated with 5 × 10^5 transduction units (in 50 μl HBSS) PKCδ shRNA Lv or control shRNA Lv on day 3 of infection by s.c. injection in same hind footpad in which *L. major* infection was given. Some mice were treated with anti-CD40 (50 μg per mouse, i.p.) for 3 alternate days beginning on the fourth day of infection. Disease severity was assessed by weekly measurement of footpad thickness for 5 wk (A) and lymph node parasite burden after 5 wk on sacrifice (A). (A, inset) Immunoblot analysis of expression of PKCδ from footpad lesion and lymph node of *L. major*-infected BALB/c mice treated with control shRNA Lv and PKCδ shRNA Lv after 5 wk infection. (B) BALB/c mice infected s.c. on one hind footpad with 2 × 10^6 *L. major* promastigotes were treated with 5 × 10^5 transduction units (in 50 μl HBSS) PKCζ shRNA Lv or control shRNA Lv on day 3 of infection by s.c. injection in the same hind footpad in which *L. major* infection was given. Some mice were treated with anti-CD40 (50 μg per mouse, i.p.) for 3 alternate days beginning on the fourth day of infection. Disease severity was assessed by weekly measurement of footpad thickness for 5 wk and lymph node parasite burden after 5 wk on sacrifice. (B, inset) Immunoblot analysis of PKCζ expression from footpad lesion and lymph node of *L. major*-infected BALB/c mice treated with control shRNA Lv and PKCζ shRNA Lv after 5 wk infection. (C) Lymph node cells from various mice, as indicated, in each group (n = 8 mice/group) were pooled and stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (2 μg/ml) for 60 h. Culture supernatants from various groups were assessed for IFN-γ and IL-4 production by ELISA. (C) Lymph node cells from various mice, as indicated, in each group (n = 8 mice/group) were pooled and stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (2 μg/ml) for 60 h. Culture supernatants from various groups were assessed for IFN-γ and IL-4 production by ELISA. The experiments were repeated twice. The error bars represent mean ± SEM with n = 8 mice/group. *p < 0.01, **p < 0.001.
disease-protective effect of PKCδ or PKCζ shRNA Lv treatment is really associated with CD40. We found that PKCδ or PKCζ shRNA Lv treatment did not have any significant effect on disease progression in CD40 knockout (CD40<sup>−/−</sup>) mice (BALB/c background), as evidenced by unchanged footpad thickness and lymph node parasite load (Fig. 7). This indicates that the disease-protective effect of PKCδ or PKCζ shRNA Lv treatment is associated with CD40 and requires its presence.

**Discussion**

Responsiveness of a receptor to stimuli depends on various signaling intermediates that act together in an integrated manner to bring a cellular response. CD40, a transmembrane receptor, regulates a wide range of immune responses and is the only known receptor for which the reciprocity, a unique property regulating cellular responsiveness, has been worked out (10–12, 33, 34). Although the CD40 signaling cascade is being revealed, the roles of PKC isoforms have remained undefined in CD40 signaling. Albeit controversial, PKC is implicated in CD40 signaling in B cells (13–16). In human monocytes, CD40 stimulation results in increased total PKC activity and translocation from cytosol to membrane (17). Members of the PKC family are involved actively in regulation of macrophage functions necessary for host defense or susceptibility to bacterial infections (21, 35). In *Leishmania* infection, an impairment of PKC-dependent protein phosphorylation of PKC substrate proteins (36), reduced expressions of MARCKS-related protein (37), c-fos, and TNF-α (18), impaired oxidative burst (38), and phagosomal maturation are reported (20). Impaired PKCβ but enhanced PKCζ expressions in *L. donovani*-infected macrophages, albeit studied very restrictively, were reported previously (19). These studies were with respect to total PKC activation and failed to imply whether *Leishmania* infection modulates the receptor-triggered effector functions regulated by PKC isoforms in macrophages. Thus, the significance of PKC isoforms in maintaining cellular responsiveness remained unsolved. Using the reciprocal CD40 signaling and the *L. major* infection as models, we have addressed the nonredundancy among PKC isoforms.

Of the eight PKC isoforms expressed in macrophages (31, 32), we show that *L. major* infection of macrophages for 72 h resulted in decreased expression of PKCδ, βI, and βII isoforms, whereas expression of PKCδ, ζ, and λ isoforms was increased. *L. major* infection differentially regulated CD40 dose-dependent phosphorylation and membrane translocation of PKC isoforms. We observed that maximum phosphorylation and membrane translocation of PKCδ, β, and ε in uninfected macrophages occurred at 6 μg/ml anti-CD40 Ab dose and that *L. major* infection impaired phosphorylation as well as membrane translocation of these PKC isoforms. In the case of PKCδ and ζ/λ, in comparison with uninfected macrophages, phosphorylation and membrane translocation were enhanced with *L. major* infection; maximum phosphorylation and membrane translocation were obtained with 1 μg/ml anti-CD40 Ab. The increased expression and CD40-induced phosphorylation and membrane translocation of PKCδ and ζ/λ in *L. major* infection suggest a proparasitic role for these PKC isoforms.

We have shown previously that CD40 stimulation at high doses results in p38MAPK phosphorylation, whereas at low doses it results in ERK1/2 phosphorylation (11). CD40-induced p38MAPK activation results in the production of IL-12, a host-protective cytokine, whereas ERK1/2 activation results in IL-10, a disease-promoting cytokine. *L. major* infection skews CD40 signaling toward ERK1/2 activation, inducing IL-10, while inhibiting activation of p38MAPK and IL-12 expression (11). CD40-induced phosphorylation and membrane translocation profiles of PKCα,

![FIGURE 7. PKCδ or PKCζ shRNA Lv treatment failed to rescue *L. major* infection in CD40-deficient mice.](http://www.jimmunol.org/)

![FIGURE 8. Proposed model showing reciprocal regulation of CD40 signaling by PKC isoforms in *L. major* infection. In BALB/c macrophages, higher doses of CD40 stimulation result in activation of PKCα, βI, βII, and ε, whereas lower doses of CD40 stimulation activate PKCδ and ζ/λ. Infection of macrophages with the protozoan parasite *L. major* impairs PKCα, βI, βII, and ε isoforms but enhances PKCδ and ζ/λ isoforms. PKCα, βI, βII, and ε isoforms regulate CD40-induced p38MAPK phosphorylation, as well as IL-12 and iNOS2 expression, and mediate a Th1 type of immune response and are essential for host protection. PKCδ and ζ/λ isoforms promote ERK1/2 phosphorylation, IL-10 production, and a Th2 immune response favoring parasite growth. In *L. major* infection, CD40 signaling is skewed toward PKCδ and ζ/λ, resulting in ERK1/2-dependent IL-10 production and a Th2 type of immune response favoring disease progression.](http://www.jimmunol.org/)

**FIGURE 8.** Proposed model showing reciprocal regulation of CD40 signaling by PKC isoforms in *L. major* infection. In BALB/c macrophages, higher doses of CD40 stimulation result in activation of PKCα, βI, βII, and ε, whereas lower doses of CD40 stimulation activate PKCδ and ζ/λ. Infection of macrophages with the protozoan parasite *L. major* impairs PKCα, βI, βII, and ε isoforms but enhances PKCδ and ζ/λ isoforms. PKCα, βI, βII, and ε isoforms regulate CD40-induced p38MAPK phosphorylation, as well as IL-12 and iNOS2 expression, and mediate a Th1 type of immune response and are essential for host protection. PKCδ and ζ/λ isoforms promote ERK1/2 phosphorylation, IL-10 production, and a Th2 immune response favoring parasite growth. In *L. major* infection, CD40 signaling is skewed toward PKCδ and ζ/λ, resulting in ERK1/2-dependent IL-10 production and a Th2 type of immune response favoring disease progression.
βI, βII, and ε are similar to those of CD40-induced p38MAPK activation, whereas PKCδ and ζ/λ activation profiles match with ERK1/2 activation, suggesting a functional correlation between PKC isoforms and these two MAPKs. Indeed, the siRNA and inhibitor experiments revealed the causal relationship between the PKC isoforms and the MAPKs. Uniquely, the PKC isoforms do reciprocally regulate CD40-induced activation of ERK1/2 and p38MAPK and production of IL-10 and IL-12: inhibition of PKCo, β, and ε resulted in the inhibition of p38MAPK phosphorylation and IL-12 production but enhancement of ERK1/2 phosphorylation and IL-10 production; in contrast, PKCδ and ζ/λ inhibition impaired ERK1/2 phosphorylation and IL-10 production but enhanced p38MAPK phosphorylation and IL-12 production. These observations support the reciprocal contribution of PKC isoforms in CD40 signaling.

Induction of IL-12 and iNOS2 in leishmaniasis is associated with host-protective function, whereas induction of IL-10 is associated with disease-exacerbating function (21, 22, 39). We have shown in this study that inhibition of PKCδ and ζ/λ enhanced the anti-leishmanial effect of CD40, whereas inhibition of PKCo, β, and ε abrogated the anti-leishmanial effect of CD40. The inhibition of the former PKC isoforms reduced amastigote number and enhanced iNOS2 expression, whereas the inhibition of the latter PKC isoforms had opposite effects. Further treatment of BALB/c mice with the lentivirally expressed PKCδ and PKCζ shRNA significantly enhanced the host-protective effect of the anti-CD40 Ab in our L. major infection model. The effect was accompanied by an IFN-γ-dominated response, suggesting that PKCδ or PKCζ shRNA treatment was associated with the restoration of Th1 response, which is required for healing and parasite clearance (40). The host-protective effect of PKCδ or PKCζ shRNA was not observed in CD40-deficient mice, suggesting that the presence of CD40 is required for the observed host-protective effect of PKCδ or PKCζ shRNA. Thus, to our knowledge, our data define for the first time the differential role of PKC isoforms in CD40 signaling in L. major infection, suggesting a novel parasite-devised fitter PKC-targeted immune evasion strategy and presenting a PKC-targeted immunotherapeutic strategy (Fig. 8).

Because calcium is a cofactor for PKCδ, βI, and εI, and the increased intracellular calcium concentration in Leishmania-infected macrophages (41, 42) might imply an enhanced acti-

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zymeinhibition of PKCδ and ζ/λ isozymes. Indeed, β-methyl-cyclodextrin-mediated cholesterol depletion resulted in the inhibition of CD40-mediated phosphorylation of PKCδ, βI, βII, and ε isoforms but enhanced phosphorylation of PKCδ and ζ/λ isozymes (data not shown). Although the possible regulation of PKCδ and ζ/λ isoforms by CD40 signaling from cholesterol-poor domains implies a novel pathway for differential activation of PKC isoforms, the restoration of host-protective immune response by targeting these isoforms provides the proof of a novel immu

notherapeutic principle.
Supplementary Figure legends

**Supplementary Figure 1.** Densitometry of the data shown in figure 1C and 1D. **A,** Densitometry plots of figure 1C showing CD40 induced phosphorylation of various PKC isoforms at indicated doses of anti-CD40 antibody stimulation in Uninfected(UIM) and *L. major* infected(IM) macrophages. **B,** Densitometry plots of figure 1D showing CD40 induced membrane translocation of various PKC isoforms at indicated doses of anti-CD40 antibody stimulation in Uninfected(UIM) and *L. major* infected(IM) macrophages.

**Supplementary Figure 2.** Densitometry of the data shown in figure 2A and 2C-G. **A,** Densitometry plots of figure 2A showing effect of indicated PKC inhibitors on CD40 induced phosphorylation of p38MAPK and ERK1/2. **B,** Densitometry plots of figure 2C-G showing effect of indicated PKC siRNAs on CD40 induced phosphorylation of p38MAPK and ERK1/2.
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